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(54) Title: EXONUCLEASE-MEDIATED NUCLEIC ACID REASSEMBLY IN DIRECTED EVOLUTION

(57) Abstract

This invention provides methods of obtaining novel polynucleotides and encoded polypeptides by the use of non–stochastic methods of directed evolution (DirectEvolution™). A particular advantage of exonuclease–mediated reassembly methods is the ability to reassemble nucleic acid strands that would otherwise be problematic to chimerize. Exonuclease–mediated reassembly methods can be used in combination with other mutagenesis methods provided herein. These methods include non–stochastic polynucleotide site–saturation mutagenesis (Gene Site Saturation Mutagenesis™) and non–stochastic polynucleotide reassembly (GeneReassembly™). This invention provides methods of obtaining novel enzymes that have optimized physical and/or biological properties. Through use of the claimed methods, genetic vaccines, enzymes, small molecules, and other desirable molecules can be evolved towards desirable properties. For example, vaccine vectors can be obtained that exhibit increased efficacy for use as genetic vaccines. Vectors obtained by using the methods can have, for example, enhanced antigen expression, increased uptake into a cell, increased stability in a cell, ability to tailor an immune response, and the like. Furthermore, this invention provides methods of obtaining a variety of novel biologically active molecules, in the fields of antibiotics, pharmacotherapeutics, and transgenic traits.

CLAIMS

What is claimed is:

1. A method for producing a mutagenized progeny polynucleotide, comprising:

(a) subjecting a starting or parental polynucleotide set to an in vitro exonuclease-mediated reassembly process so as to produce a progeny polynucleotide set;

whereby the exonuclease-mediated reassembly process is exemplified, in a non-limiting fashion, by subjection to a 3' exonuclease treatment, such as treatment with exonuclease III, which acts on 3' underhangs and blunt ends, to liberate 3'-terminal but not 5'-terminal nucleotides from a starting double stranded polynucleotide, leaving a remaining strand that is partially or completely free of its original partner so that, if desired, the remaining strand may be used to achieve hybridization to another partner;

whereby the exonuclease-mediated reassembly process is further exemplified, in a non-limiting fashion, by subjection to a 5' exonuclease treatment, such as treatment with red alpha gene product, that acts on 5' underhangs to liberate 5'-terminal nucleotides from a starting double stranded polynucleotide, leaving a remaining strand that is partially or completely free of its original partner so that, if desired, the remaining strand may be used to achieve hybridization to another partner;

whereby the exonuclease-mediated reassembly process is further exemplified, in a non-limiting fashion, by subjection to an exonuclease treatment, such as treatment with Mung Bean Nuclease or treatment with S1 Nuclease or treatment with E.coli DNA Polymerase, that acts on overhanging ends, including on unhybridized ends, to liberate terminal nucleotides from an unhybridized single-stranded end of an annealed nucleic acid strand in a heteromeric nucleic acid complex, leaving a shortened but hybridized end to

facilitate polymerase-based extension and/or ligase-mediated ligation of the treated end;

and whereby the exonuclease-mediated reassembly process is also exemplified by a dual treatment, that can be performed, for example, non-simultaneously, with both an exonuclease that liberates terminal nucleotides from underhanging ends or blunt ends as well as an exonuclease that liberates terminal nucleotides from overhanging ends such as unhybridized ends.

2. The method of claim 1 for producing a mutagenized progeny polynucleotide, wherein the step of (a) subjecting a starting or parental polynucleotide set to an in vitro exonuclease-mediated reassembly process so as to produce a progeny polynucleotide set; is comprised of:

(i) subjecting a starting or parental polynucleotide set to a 3' exonuclease treatment that acts on 3' underhangs and blunt ends, to liberate 3'-terminal but not 5'-terminal nucleotides;

whereby said 3' exonuclease is exemplified, in a non-limiting fashion, by treatment with an exonuclease, such as exonuclease III, to liberate 3'-terminal but not 5'-terminal nucleotides from a starting double stranded polynucleotide, leaving a remaining strand that is partially or completely free of its original partner so that, if desired, the remaining strand may be used to achieve hybridization to another partner.

3. The method of claim 1 for producing a mutagenized progeny polynucleotide, wherein the step of (a) subjecting a starting or parental polynucleotide set to an in vitro exonuclease-mediated reassembly process so as to produce a progeny polynucleotide set; is comprised of:

(i) subjecting a starting or parental polynucleotide set to a 5' exonuclease treatment that acts on 5' underhangs to liberate 5'-terminal nucleotides;

whereby said 5' exonuclease is exemplified, in a non-limiting fashion, by treatment with an exonuclease, such as red alpha gene product, to liberate 5'-terminal nucleotides from a starting double stranded polynucleotide, leaving a remaining strand that is partially or completely free of its original partner so that, if desired, the remaining strand may be used to achieve hybridization to another partner.

4. The method of claim 1 for producing a mutagenized progeny polynucleotide, wherein the step of (a) subjecting a starting or parental polynucleotide set to an in vitro exonuclease-mediated reassembly process so as to produce a progeny polynucleotide set; is comprised of:

(i) subjecting a starting or parental polynucleotide set to an exonuclease treatment that liberates terminal nucleotides from nucleic acid overhangs;

whereby said treatment is exemplified, in a non-limiting fashion, by subjection to an exonuclease treatment, such as treatment with Mung Bean Nuclease or treatment with S1 Nuclease or treatment with E.coli DNA Polymerase, that acts on overhanging ends, including on unhybridized ends, to liberate nucleotides from an unhybridized single-stranded end of an annealed nucleic acid strand in a heteromeric nucleic acid complex, leaving a shortened but hybridized end to facilitate polymerase-based extension and/or ligase-mediated ligation of the treated end.

5. The method of claim 1 for producing a mutagenized progeny polynucleotide, wherein the step of (a) subjecting a starting or parental polynucleotide set to an in vitro exonuclease-mediated reassembly process so as to produce a progeny polynucleotide set; is comprised of:

(i) subjecting a starting or parental polynucleotide set to a 3' exonuclease treatment that acts on 3' underhangs and blunt ends, to liberate 3'-terminal but not 5'-terminal nucleotides; and

(ii) subjecting a starting or parental polynucleotide set to an exonuclease treatment that liberates terminal nucleotides from nucleic acid overhangs;

whereby the exonuclease-mediated reassembly process is comprised of a dual treatment, that can be performed, for example, non-simultaneously, with both an exonuclease that liberates terminal nucleotides from underhangs or blunt ends as well as an exonuclease that liberates terminal nucleotides from overhangs such as unhybridized ends.

6. The method of claim 1 for producing a mutagenized progeny polynucleotide, wherein the step of (a) subjecting a starting or parental polynucleotide set to an in vitro exonuclease-mediated reassembly process so as to produce a progeny polynucleotide set; is comprised of:

(i) subjecting a starting or parental polynucleotide set to a 5' exonuclease treatment that acts on 5' underhangs to liberate 5'-terminal nucleotides; and

(ii) subjecting a starting or parental polynucleotide set to an exonuclease treatment that liberates terminal nucleotides from nucleic acid overhangs;

whereby the exonuclease-mediated reassembly process is comprised of a dual treatment, that can be performed, for example, non-simultaneously, with both an exonuclease that liberates terminal nucleotides from underhangs or blunt ends as well as an exonuclease that liberates terminal nucleotides from overhangs such as unhybridized ends.

7. A method for producing a mutagenized progeny polynucleotide having at least one desirable property comprised of the step of:

(a) subjecting a starting or parental polynucleotide set to an in vitro exonuclease-mediated reassembly process so as to produce a progeny polynucleotide set; and

(b) subjecting the progeny polynucleotide set to an end selection-based screening and enrichment process, so as to select for a desirable subset of the progeny polynucleotide set;

whereby the above steps can be performed iteratively and in any order and in combination;

whereby the end selection-based process creates ligation-compatible ends;

whereby the creation of ligation-compatible ends is optionally used to facilitate one or more intermolecular ligations, that are preferably directional ligations, within members of the progeny polynucleotide set so as to achieve assembly &/or reassembly mutagenesis;

whereby the creation of ligation-compatible ends serves to facilitate ligation of the progeny polynucleotide set into an expression vector system and expression cloning;

whereby the expression cloning of the progeny polynucleotide set serves to generate a polypeptide set;

whereby the generated polypeptide set can be subjected to an expression screening process; and

whereby expression screening of the progeny polypeptide set provides a means to identify a desirable species, e.g. a mutant polypeptide or alternatively a polypeptide fragment, that has a desirable property, such as a specific enzymatic activity.

8. A method for generating a mutagenized progeny polynucleotide from a collection of progenitor polynucleotides, comprising:

a) annealing a poly-binding nucleic acid strand to two mono-binding nucleic acid strands to generate an annealed heteromeric complex of nucleic acid strands;

wherein the poly-binding nucleic acid strand and the two mono-binding nucleic acid strands are each derived from a different molecular species in said collection of progenitor polynucleotides;

wherein the said collection of progenitor polynucleotides is preferably comprised of nonidentical though possibly related progenitor polynucleotides, as exemplified by a collection of genes encoding dehalogenases;

and wherein the poly-binding nucleic acid strand to two mono-binding nucleic acid strands each have at least a 7 nucleotide-long sequence of identity to the progenitor polynucleotides from which it is derived;

b) and subjecting the unhybridized single-stranded ends of the annealed mono-binding nucleic acid strands in the heteromeric complex to an exonuclease treatment that degrades said unhybridized ends;

whereby the annealment of working poly-binding and mono-binding strands derived from nonidentical polynucleotides thus allows one to generate a chimerization of said nonidentical polynucleotides;

whereby, in a library of said annealed complexes of nucleic acid strands, many component strands have unhybridizable ends that are suboptimal or not serviceable for priming polymerase-based extension; and

whereby the exonuclease treatment removes such unhybridizable ends to convert the annealed complexes of nucleic acid strands into better primers for polymerase-based extension.

9. The method of claim 8, further comprising the step of:

c) subjecting the annealed heteromeric complex to polymerase-based extension.

10. The method of claim 9, further comprising the step of:

d) subjecting the annealed nucleic acid strands to a ligase treatment;

whereby subjection to ligase treatment is exemplified by subjection to T4 DNA Ligase treatment to achieve intermolecular ligation between the two annealed mono-binding strands, which thus become covalently linked forming a chimerized strand.

11. The method of claim 10, further comprising the step of:

e) separating the poly-binding nucleic acid strand from the ligated mono-binding nucleic acid strands;

whereby the separation of a poly-binding nucleic acid strand from ligated mono-binding nucleic acid strands to which it is annealed can be achieved, for example, by either denaturation or by exposure to an enzymatic activity that selectively acts on the poly-binding nucleic acid strands.

12. The method of claim 11, further comprising the step of:

f) generating a nucleic acid strand that is complementary to the ligated mono-binding nucleic acid strand;

whereby the resultant product is comprised of a double stranded mutagenized progeny polynucleotide.

13. The method of any of claims 1-12 wherein the mutagenized progeny polynucleotide is a gene or gene pathway.

14. The method of 13 further comprising:

expressing the generated mutagenized progeny polypeptide in a suitable host;

whereby said expression leads to the generation of a product of the polypeptide that can be detected by expression screening.